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ORIGINAL RESEARCH PAPER

ANTIOXIDANT, ANTIHYPERTENSIVE AND CELL REGULATION ACTIVITY OF BIOACTIVE PEPTIDES FROM GOAT COLOSTRUM FERMENTED WITH MIXED KEFIR CONSORTIA FOR HEALTH BENEFITS

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Abstract: The aim of this work was to investigate the effect of goat colostrum fermentation on the isolation of bioactive peptides and their biochemical and biological properties. In this respect, goat colostrum was fermented using kefir and mixed microbial consortia, followed by centrifugal ultrafiltration to obtain peptide fractions with the molecular weight (MW) less than 3 kDa. The results showed that fermentation increased the soluble protein content up to 2.7-fold, compared to unfermented colostrum. Electrophoretic analysis confirmed casein degradation and enrichment in low MW peptides. The peptide fractions exhibited enhanced antioxidant activity and significant angiotensin-converting enzyme (ACE) inhibitory activity. *In vitro* cytocompatibility testing showed stimulation of L929 fibroblasts metabolic activity. These findings supported mixed microbial fermentation as a promising approach for obtaining bioactive peptides of interest in functional food development.

Keywords: *angiotensin-converting enzyme, bioactive peptides, colostrum, cytocompatibility, fermentation, free radical scavenging*

INTRODUCTION

Milk and colostrum are recognized as valuable sources of proteins and peptides with important nutritional, functional, and biological properties, providing essential amino acids and a wide range of bioactive compounds [1]. During the last decade, significant advances have been achieved in technologies aimed at the separation, fractionation, and purification of milk-derived proteins, particularly from bovine colostrum and whey. Industrial-scale processes have been developed for the isolation of major whey proteins such as immunoglobulins, lactoferrin, lactoperoxidase, α-lactalbumin, and β-lactoglobulin [2].

Colostrum is distinguished from mature milk by its markedly higher concentration of bioactive proteins, especially immunoglobulins, lactoferrin, and growth factors, reflecting its essential role in neonatal nutrition and immune protection [3]. Beyond intact proteins, peptides derived from milk proteins exhibit specific biological activities, making them promising ingredients for functional foods and nutraceutical applications. These properties are attributed both to native protein structures and to physiologically active peptides encrypted within protein sequences and released during enzymatic hydrolysis or microbial fermentation [4].

Among bioactive peptides, antioxidant peptides play a crucial role in maintaining redox balance by preventing free radical formation or scavenging reactive oxygen species, thereby limiting oxidative damage to biomolecules and reducing the risk of oxidative stress-related disorders [5]. In addition, bioactive peptides may exert a wide range of physiological effects *in vivo*, influencing gastrointestinal, cardiovascular, endocrine, immune, and nervous systems [6]. In particular, peptides with antihypertensive activity, mainly through angiotensin-converting enzyme (ACE) inhibition, have attracted considerable attention due to their potential role in cardiovascular health [7].

Fermented dairy products represent an important source of bioactive peptides as a result of the proteolytic activity of microorganisms involved in fermentation processes [8]. Numerous studies have reported the generation of peptides with antioxidant, antihypertensive, and immunomodulatory activities in fermented milk and colostrum-based matrices [9, 10]. However, despite increasing evidence supporting their health-promoting effects [11], large-scale production and commercial exploitation of bioactive peptides remain limited, mainly due to the lack of optimized and scalable technologies that ensure consistent peptide yield and bioactivity.

Although bovine colostrum has been extensively investigated, goat colostrum remains comparatively underexplored despite its distinctive protein composition and high biological value. Goat milk exhibits a different casein-to-whey protein ratio and a favorable digestibility profile, which may enhance the generation and bioavailability of bioactive peptides [12]. Innovative fermentation strategies are therefore required to improve peptide release while preserving the natural complexity of the colostrum matrix.

The aim of this study was to investigate the production of bioactive peptides from goat colostrum fermented with different microbial consortia. Fermentations were performed using kefir, kombucha, kefir-yeast and kefir-kombucha starter cultures. The obtained peptide fractions were characterized in terms of antioxidant, antihypertensive and cell regulation activities, to evaluate their potential application as functional food ingredients.

MATERIALS AND METHODS

Materials

Goat colostrum was kindly provided by Gruiu farm, Calarasi, Romania. Kefir grains and *Candida lipolytica* MIUG D67 yeast were kindly provided from the Microorganism Collection of "Dunărea de Jos" University of Galati, Romania. Kombucha symbiotic culture was kindly provided by Laboratoarele Medica SRL, Otopeni, Romania. Murine fibroblast NCTC clone L929 and HT-29 cell lines were purchased from ECACC (Sigma-Aldrich, Germany). Minimum Essential Medium (MEM), fetal bovine serum (FBS), penicillin–streptomycin–neomycin mixture (PSN) were purchased from Sigma-Aldrich (Germany). Bicinchoninic acid (BCA), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), angiotensin-converting enzyme (ACE), hippuryl-L-histidyl-L-leucine (HHL) and all other chemical reagents were of analytical grade purity and purchased from Sigma-Aldrich (Germany), unless otherwise specified.

Fermentation of goat colostrum

Before fermentation, frozen goat colostrum was thawed at 4 °C and homogenized. Samples were prepared at a concentration of 8 % (w/v) and subjected to heat treatment at 105 °C, for 10 min, in order to reduce the initial microbial load. After cooling to room temperature, the substrate was used for inoculation. Fermentation was performed under static conditions, in sterile 100 mL containers. Goat colostrum was subjected to fermentation using four different inoculation systems, as summarized in Table 1.

Table 1. *Experimental design of goat colostrum fermentation variants*

Sample abbreviation	Inoculation system
Control	Unfermented goat colostrum
FC1	Goat colostrum (8 %, w/v) fermented with 2.5 % (w/v) kefir grains
FC2	Goat colostrum (8 %, w/v) fermented with 1 % (w/v) *Candida lipolytica* MIUG D67 (2.15×10^7 CFU·mL^{-1}) in combination with 0.1 % (w/v) kefir grains
FC3	Goat colostrum (8 %, w/v) fermented with 2.5 % (v/v) kombucha culture
FC4	Goat colostrum (8 %, w/v) fermented with 1.25 % (w/v) kefir grains in combination with 1.25 % (v/v) kombucha culture

Preparation of bioactive peptide fractions

Fermented samples were extracted in distilled water, at 4 °C, overnight (Figure 1).
The suspensions were subsequently centrifuged at 1300 × g, for 10 min, and the resulting supernatant was subjected to centrifugal ultrafiltration using Amicon Ultra filter units with a molecular weight cut-off (MWCO) of 3 kDa, according to the manufacturer's instructions. Aliquots of 2 mL were loaded into the filter devices and centrifuged at 5000 × g, for 20 min, at room temperature. The obtained filtrate represented a peptide-enriched fraction containing molecules with the molecular weight (MW) less than 3 kDa.

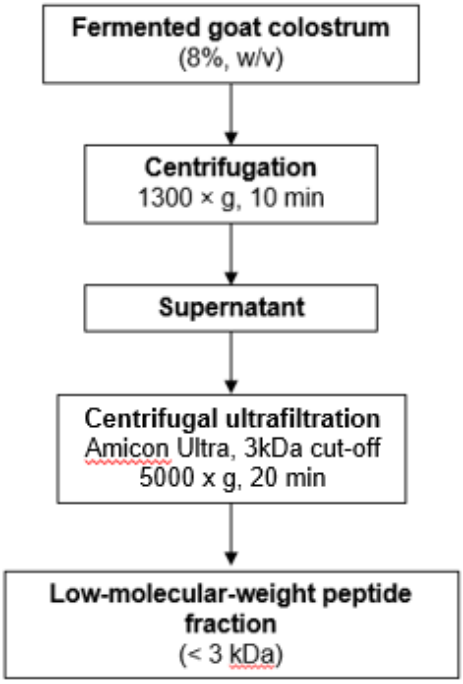


Figure 1. *Schematic representation of the preparation of peptide fractions with the molecular weight less than 3 kDa from fermented goat colostrum by centrifugal ultrafiltration*

Determination of protein content

Protein concentration was determined using the BCA assay according to Arslan *et al.* [13]. The BCA working reagent was prepared by mixing bicinchoninic acid solution with 4 % $CuSO_4$ solution in a 50:1 (v/v) ratio. A volume of 20 µL of sample was mixed with 160 µL of BCA reagent. The reaction mixture was incubated for 30 min, at 60 °C. Absorbance was measured at 562 nm using a SPECTROstar Nano microplate reader (BMG Labtech, Germany). A calibration curve was built using bovine serum albumin (BSA) in the concentration range of 0.05 - 2 $mg \cdot mL^{-1}$.

Electrophoretic analysis of bioactive peptides

Colostrum, fermented colostrum and peptide fractions with the MW less than 3 kDa were analyzed using 10 - 20 % Novex Tricine–SDS–polyacrylamide gradient gels (Thermo Fisher Scientific, USA), in a vertical electrophoresis unit (Biometra, Germany), according to Schägger [14]. Samples were diluted in Novex™ Tricine-SDS sample buffer supplemented with NuPAGE™ sample reducing agent (Thermo Fisher Scientific, USA). The mixtures were heated at 85 °C, for 3 min, prior to loading onto gradient gels, together with a low MW marker (1.7 - 40 kDa) (Sigma-Aldrich, Germany). Electrophoresis was performed at 30 V, for 30 min, followed by 90 V, for 2 h. After migration, gels were stained overnight with Roti® Blue staining solution (Carl Roth, Germany) and destained using a methanol/water mixture (1:3, v/v). Gel images were acquired using a gel documentation system (Vilber Lourmat, Germany).

Determination of antioxidant activity

The antioxidant activity of the samples was evaluated using the Trolox equivalent antioxidant capacity (TEAC) assay based on the inhibition of $ABTS^{\bullet+}$ radical cation formation [15]. The ABTS radical cation was generated by mixing a 7 mM ABTS solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) and incubating the mixture for 12 - 16 h, at room temperature, in the dark. Prior to analysis, the resulting solution was diluted with distilled water to obtain an absorbance of 0.700 ± 0.02 at 734 nm. A volume of 100 µL of sample was mixed with 1 mL of ABTS solution, and incubated, at room temperature, for 6 min. A positive control of 1 mM ascorbic acid was similarly processed. The absorbance was measured at 734 nm using an UV–Vis spectrophotometer (Jasco V-650, Japan). A Trolox solution in the concentration range of 0 - 250 µM was used to generate the calibration curve. Results were expressed as mM Trolox equivalents per gram dry weight (mM TE/g d.w.).

Determination of ACE inhibitory activity

The ACE inhibitory activity was determined according to the method previously described by Ibrahim *et al*. [7]. Briefly, 50 µL of sample were mixed with 180 µL of 5 mM HHL dissolved in 100 mM sodium borate buffer containing 300 mM NaCl (*p*H 8.3). After pre-incubation for 5 min, 20 µL of ACE solution (2 mU) were added, and the reaction mixture was incubated at 37 °C for 90 min. The reaction was terminated by adding 250 µL of 1 N HCl. The hippuric acid formed was extracted with 1.7 mL of ethyl acetate. The organic phase was evaporated at 100 °C for 15 min, and the residue was dissolved in 1 mL of distilled water. A positive control using 35 nM captopril was similarly processed. Absorbance was measured at 228 nm using an UV-Vis spectrophotometer (Jasco V-650, Japan). ACE inhibitory activity was expressed as inhibition percentage (%).

***In vitro* cytocompatibility testing**

Cell viability and proliferation were evaluated on murine fibroblast-like NCTC clone L929 cell line. Cells were seeded in 96-well plates at a density of 4×10^4 cells·mL^{-1} and cultured overnight in MEM supplemented with 10 % FBS and 1 % PSN, at 37 °C in a humidified atmosphere containing 5 % CO_2. After cell attachment, the culture medium was replaced with fresh medium containing the test samples at concentrations ranging from 0.25 to 2 mg·mL^{-1}. Cells were further incubated for 48 h. Cell viability was assessed using the colorimetric MTT assay [16]. Briefly, the culture medium was replaced with MTT solution at a final concentration of 0.25 mg·mL^{-1} and incubated for 3 h. After incubation, the MTT solution was removed, and an equal volume of isopropanol was added to dissolve the formazan crystals. Plates were placed on a shaker, for 15 min, to ensure complete solubilization. Untreated cells served as control. All samples were tested in triplicate. Absorbance was measured at 570 nm using a microplate reader (SpectroStar Nano, BMG Labtech, Germany). Cell viability (%) was calculated as percentage from control cells, considered 100 % viable.

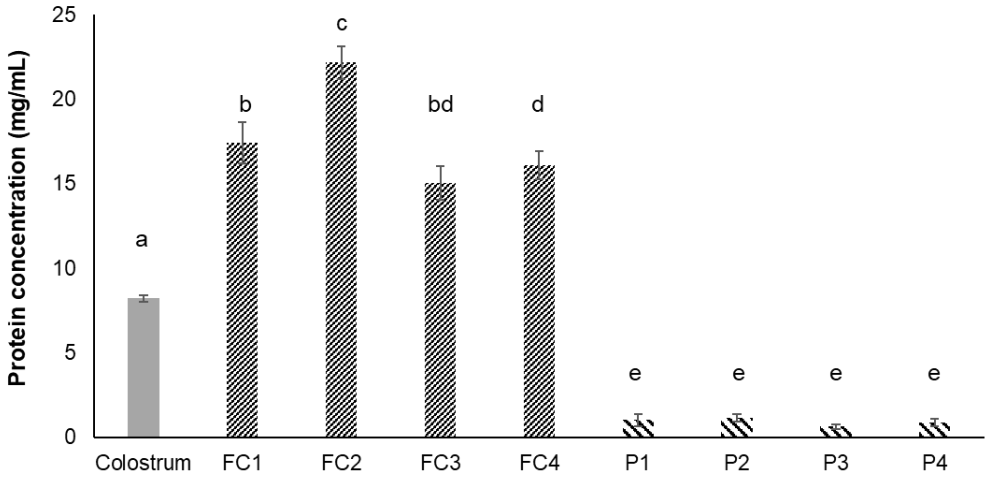
Statistical analysis

All experiments were performed in triplicate (n = 3). Results are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using one-way ANOVA analysis of variance (Microsoft Excel v. 11), followed by Tukey's post hoc test for multiple comparisons (www.statscalculators.com). For pairwise comparisons between control and treated samples, Student's *t*-test (Microsoft Excel v. 11) was applied. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Analysis of protein content

Goat colostrum, presenting a rich content of proteins and polypeptides, was fermented by adding different starter consortia of kefir (FC1), kombucha (FC3) and mixed consortia of kefir-yeast (FC2) and kefir-kombucha (FC4). Due to cell lysis and proteolysis during fermentation, the protein content increased up to 2.7 times, compared to unfermented colostrum (Figure 2).



The results were expressed as mean ± SD (n = 3) and analyzed by ANOVA. Values that share the same letter are not significantly different, as determined by Tukey's post hoc test for $p < 0.05$

Figure 2. *Protein content values in goat colostrum fermented with different microbial consortia of kefir (FC1), kefir-yeast (FC2), kombucha (FC3) and kefir-kombucha (FC4), and the corresponding fractions of bioactive peptides with the molecular weight less than 3 kDa (P1 - P4)*

The data showed that the value of 8.21 mg·mL^{-1} protein content in goat colostrum increased 1.8 - 2.1 times, up to 15 - 17.5 mg·mL^{-1}, in kefir (FC1) or kombucha-treated (FC3) variants, and 2 - 2.7 times, up to 16 - 22 mg·mL^{-1}, in variants fermented with mixed consortia. The highest value was recorded in kefir-yeast fermented variant (FC2), followed by kefir-kombucha fermented variant (FC4), indicating that these microbial combinations could induce the highest hydrolysis degree of goat colostrum and enrich the protein content. Separation of bioactive peptide fractions with the MW less than 3 kDa

presented a protein content varying between 0.64 mg·mL^{-1} in variant FC3 fermented with kombucha and 1.16 mg·mL^{-1} in variant FC2 fermented with kefir-yeast. The protein content of kefir and kefir-kombucha fermented variants had in between values.

Characterization of bioactive peptides by electrophoresis in Tricine-SDS-polyacrylamide gel in gradient

The proteins present in colostrum and fermented variants (FC1 – FC4) were analyzed for electrophoretic profile (Figure 3a).

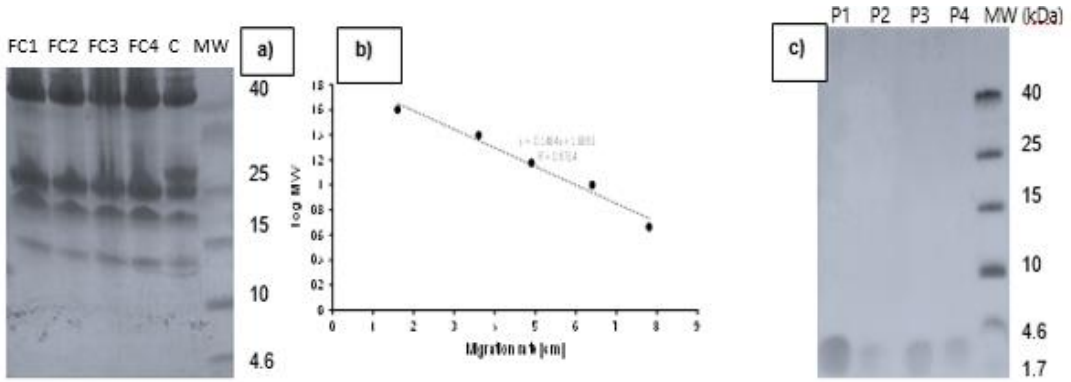


Figure 3. *Bioactive peptides analysis by gel electrophoresis: a) electrophoresis profile of goat colostrum fermented with different microbial consortia of kefir (FC1), kefir-yeast (FC2), kombucha (FC3), kefir-kombucha (FC4), unfermented colostrum (C) and a molecular weight marker (MW) in Tricine-SDS-polyacrylamide gel in gradient; b) standard curve of the logarithm of molecular weight (log MW) as a function of migration rate; c) corresponding fractions of bioactive peptides with the molecular weight less than 3 kDa (P1 - P4) and a molecular weight marker (MW), in Tricine-SDS-polyacrylamide gel in gradient*

According to the MW of proteins present in the low MW marker and their migration distance, a standard curve was built (Figure 3b), and MW of proteins from colostrum (C) and fermented colostrum variants (FC1 – FC4) were calculated (Table 2).

Table 2. *Identification of proteins from goat colostrum according to the molecular weight (MW) of corresponding bands in the electrophoretic profile*

Colostrum protein MW [kDa]	Identified protein	Theoretical MW [kDa]
63.58	Lactoferrin	80
59.18	Lactoperoxidase	78
48.12	Bovine serum albumin	66
25.29	α-Casein / β-Casein	23-25 / 24
21.34	κ-Casein	19
18.64	β-Lactoglobulin	18.4
13.28	α-Lactalbumin	14

Main proteins of colostrum (C), i.e. caseins and whey proteins, having MW below 40 kDa were identified by gel gradient electrophoresis (Figure 3a). Thus, the low MW bands observed in the electrophoretic profile corresponded to β-lactoglobulin (18.4 kDa) and α-lactalbumin (14 kDa). A doublet of prominent bands, corresponding to α- and β-caseins (24 kDa) and κ-casein (19 kDa), respectively, were also observed. One prominent band, corresponding to BSA (66 kDa), followed by other two bands with high MW, corresponding to lactoperoxidase (78 kDa) and lactoferrin (80 kDa), were observed outside the MW marker range.

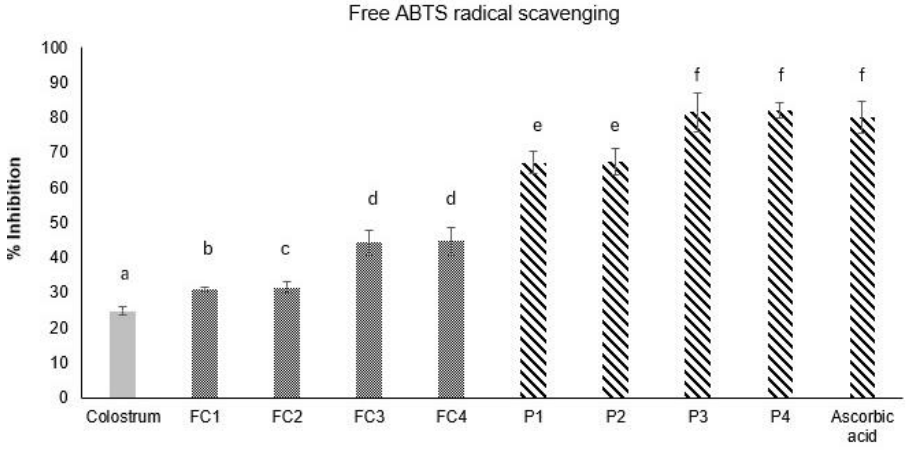
After colostrum fermentation, the band of α- and β-caseins was not observed in FC1 – FC4 variants, indicating its degradation due to proteolytic activity. Previous studies have shown that α- and β-caseins are primary substrates for enzymatic hydrolysis during fermentation, largely due to interactions with proteases from starter cultures [17 – 19]. In contrast, κ-casein, located at the surface of casein micelles, undergoes differential degradation with glycomacropeptide release, reflecting its distinct structural role in micellar stability [20, 21].

On the other hand, electrophoresis of bioactive peptides separated using membranes with MW cutoff of 3 kDa showed that all fractions (P1 - P4) contained a group of peptides with MW between 1.7 - 4.6 kDa (Figure 3c), confirming the efficiency of the fermentation process and centrifugal ultrafiltration.

Characterization of the biological activity

Analysis of free radical scavenging activity

The antioxidant activity of colostrum, fermented variants (FC1 – FC4) and bioactive peptide fractions was analyzed as their capacity to scavenge free ABTS radicals. The results are presented in Figure 4.



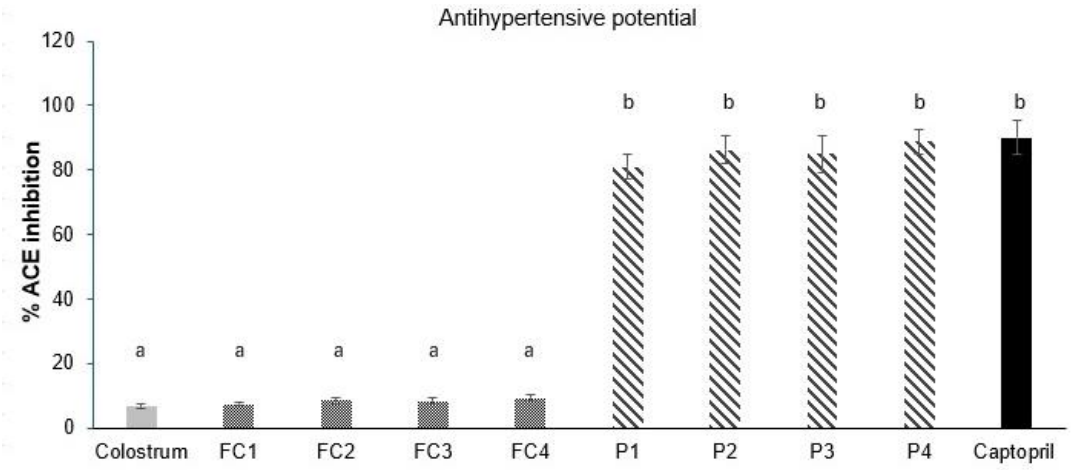
The results were expressed as mean ± SD (n = 3) and analyzed by ANOVA. Values that share the same letter are not significantly different, as determined by Tukey's post hoc test for $p < 0.05$

Figure 4. *Antioxidant activity values of goat colostrum fermented with different microbial consortia of kefir (FC1), kefir-yeast (FC2), kombucha (FC3) and kefir-kombucha (FC4), and the corresponding fractions of bioactive peptides with the molecular weight less than 3 kDa (P1 - P4), determined as free ABTS radical scavenging capacity*

The data showed that the antioxidant activity increased after fermentation of colostrum by 1.26 - 1.28 times in kefir (FC1) and kefir–yeast (FC2) fermented variants, and by 1.79 - 1.81 times in kombucha (FC3) and kefir–kombucha (FC4) fermented variants, which is consistent with previous reports indicating that fermentation enhances antioxidant capacity in dairy matrices due to microbial proteolysis [22]. Bioactive peptide fractions with the MW less than 3 kDa presented higher scavenging activity against ABTS radicals than colostrum and fermented colostrum variants, reaching 81.5 - 82 % inhibition of free ABTS radicals, values similar to 1 mM ascorbic acid (80 %), a known antioxidant agent. This observation agreed with studies demonstrating that low MW peptides exhibited stronger antioxidant activity compared to larger polypeptides [23, 24].
Moreover, it was observed that colostrum fermented with kombucha (FC3) and kefir–kombucha (FC4), and the corresponding peptide fractions, presented higher antioxidant activity than variants fermented with other starter cultures. This could be due to the presence of polyphenols derived from green tea in kombucha culture, which are known to exert strong antioxidant effects [25, 26].

Analysis of antihypertensive potential by inhibition of ACE activity

ACE plays a central role in the regulation of blood pressure by converting angiotensin I into the potent vasoconstrictor angiotensin II and by inactivating bradykinin, a vasodilatory peptide, thereby contributing to the control of vascular tone and electrolyte balance [27]. Food-derived peptides exhibiting angiotensin-converting enzyme (ACE) inhibitory activity have attracted considerable attention due to their potential to reduce hypertension and associated cardiovascular risks [8]. In this study, the ACE inhibitory activity of bioactive peptides obtained from different fermented colostrum variants was evaluated, and the results are presented in Figure 5.



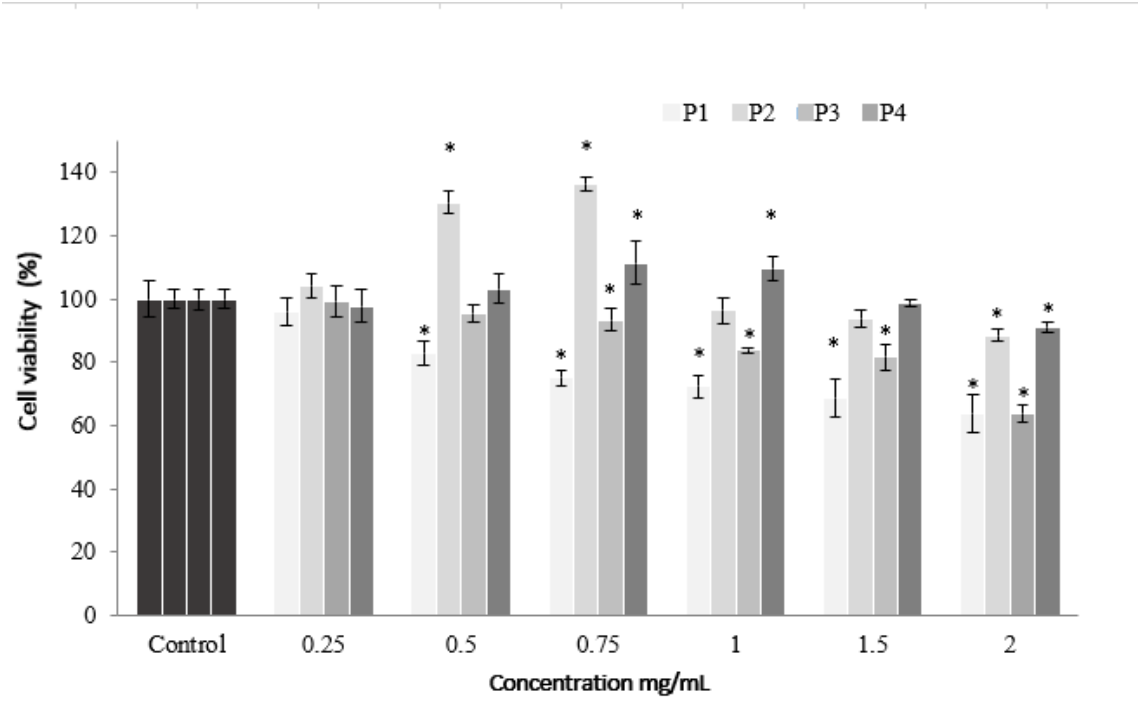
The results were expressed as mean ± SD (n = 3) and analyzed by ANOVA. Values that share the same letter are not significantly different, as determined by Tukey's post hoc test for $p < 0.05$

Figure 5. *Inhibition of angiotensin-converting enzyme (ACE) activity of goat colostrum fermented with different microbial consortia of kefir (FC1), kefir-yeast (FC2), kombucha (FC3) and kefir-kombucha (FC4), and the corresponding fractions of bioactive peptides with the molecular weight less than 3 kDa (P1 - P4)*

The data showed that only bioactive peptides with the MW less than 3 kDa presented the capacity to inhibit ACE, thus indicating an antihypertensive potential. The values ranging between 81 - 88.7 % were similar to that of 35 nM captopril (90 %), a known inhibitor of ACE. Similar results were obtained for hydrolysates of whey proteins and caseins from goat milk digested with pepsin, reaching 90 % inhibition of ACE activity [28]. They identified bioactive peptides with PEQSLACQCL, QSLVYPFTGPI and ARHPHPHLSFM sequences by MALDI-TOF MS/MS, indicating that hydrophobic amino acids and proline strongly contribute to this activity. Molecular docking studies revealed an interaction between peptides derived from whey proteins, such as IIAE, LIVTQ and LVYPFP, and the amino acids from human ACE active site, Gln 259, His 331, and Thr 358, forming strong hydrogen bonds [6].

Effect on cell metabolism in vitro

Bioactive peptides are increasingly recognized as key regulators of cellular functions, exerting modulatory effects on cell proliferation, differentiation, oxidative balance and metabolic activity [29, 30]. In the present study, goat colostrum-derived peptides obtained through fermentation with different starter cultures were evaluated for their influence on cell viability and proliferation using a stabilized murine fibroblast cell line (L929). The results are presented in Figure 6.



The results were expressed as mean ± SD (n = 3) and analyzed by Student *t*-test. *p < 0.05, compared to control

Figure 6. *Cell viability of L929 cultivated in the presence of fractions of bioactive peptides with the molecular weight less than 3 kDa (P1 - P4), for 48 h*

The results showed that L929 cell viability remained high in the presence of P2 and P4 peptide fractions, with values ranging between 88 % and 137 % across all tested concentrations (0.25 - 2 mg·mL^{-1}), confirming their cytocompatibility (cell viability > 70 %). Notably, significantly higher cell viability ($p < 0.05$) compared to the

untreated control was observed at 0.5 - 0.75 mg·mL^{-1} for P2 and 0.75 - 1 mg·mL^{-1} for P4, suggesting a stimulatory effect of these fermentation-derived peptides on cellular metabolic activity and proliferation at moderate concentrations.
The P3 fraction also demonstrated good cytocompatibility over a broad concentration range (0.25 - 1.5 mg·mL^{-1}), with cell viability values between 82 % and 99 %. Similarly, P1 was cytocompatible within 0.25 - 1.5 mg·mL^{-1}; however, it induced a more pronounced dose-dependent decrease in viability, from 96 % to 69 %. At the highest tested concentration (2 mg·mL^{-1}), both P1 and P3 reduced cell viability to approximately 64 %, indicating moderate cytotoxicity under the experimental conditions. No statistically significant proliferative effect was observed for P1 and P3 fractions.
These findings are consistent with previous reports demonstrating that low MW peptides derived from milk and fermented dairy products can promote cell proliferation and exert cytomodulatory effects *in vitro*, with responses strongly influenced by peptide concentration and structural characteristics [31 – 33].

CONCLUSIONS

Fractions of bioactive peptides with the MW less than 3 kDa were successfully isolated from goat colostrum fermented with different starter cultures. The analyses demonstrated their antioxidant activity with higher values for peptides derived from kombucha and kefir–kombucha fermented goat colostrum, their antihypertensive potential through ACE inhibition, and their cytocompatibility. Fractions of peptides derived from mixed consortia fermentations of kefir-yeast and kefir-kombucha had also the ability to stimulate cellular metabolism *in vitro*. All these results demonstrated that combined kefir-yeast and kefir–kombucha fermentations represent a biotechnological approach offering synergistic proteolytic and metabolic activities derived from lactic acid bacteria, yeasts, and acetic acid bacteria. Such complex microbial consortia might favor the production of low MW peptides with enhanced biological activity. Overall, these findings support the microbial fermentation strategy as a promising approach for the production of valuable bioactive ingredients with potential physiological and metabolic benefits, contributing to the development of novel functional foods for human health.

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